

From The Department of Oncology-Pathology
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**The destiny of natural killer cells in the tumor
microenvironment:
To be suppressed or activated?**

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The destiny of natural killer cells in the tumor-
microenvironment:
To be suppressed or activated?

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Till minnet av Seba

”The course of nature” is a term easier for a doctor to say than a family member to understand!

My mentor says it’s not fair..... it’s never fair!

I had an aunt... she was very close friend... young and beautiful.....

One day she was diagnosed for breast cancer. For more than ten years ago it was very much as consent to death. One operation and multiple chemotherapy and radiotherapy courses but six months later brain metastases appeared and a month later she fell for her long-last sleep.

Seba was at the age of 32 when she died leaving a husband and a six years old daughter behind. “Time heals all wounds” I can’t agree. We have learned how to live without you but it’s always painful to think about you. You are there in our thoughts and you will always be there!

I promised myself to do everything in my hands to help to improve cancer therapies and I’ll always do until the last day in my life.

Dhifaf

ABSTRACT

In the mid-70s, the term “natural” was used based on the functional properties of NK cells where they recognize and lyse certain tumor cells without requirement of prior immunization. Although NK cells are “natural” in their targeting of tumors, their responses in the tumor microenvironment have been very challenging to determine. The tumor microenvironment is known to consist of a heterogeneous population of cells and secreted factors. The failure in the immune surveillance may in part be due to sustained immunological selection pressure on tumor cells resulting in the development of tumor escape variants that are effectively invisible to the immune system. On the other hand it can be due to the complex network of immune suppressive compartments in the tumor microenvironment.

We have studied NK cell activity in the tumor microenvironment. The study was divided into two parts; 1) how to augment NK cell activity against tumor cells, and 2) examine the suppression of NK cells in the tumor microenvironment.

A novel proteasome inhibitor (b-AP15) was found to sensitize tumor cells of different origin to NK cell and T cell- TRAIL mediated killing. Combined therapy with b-AP15 and infusion of NK cells and T cells in animals resulted in reduced tumor progression and prolonged survival. We also found that enhancement of TRAIL expression on NK cells could augment their cytotoxicity against tumors. Such action was possible when NK cells were interacting with monocytes that were manipulated with a bisphosphonate called Zoledronic Acid to produce IFN- γ .

Studies on suppression of NK cells involved the outcome of interaction between NK cells and dendritic cells (DC) or myeloid-derived suppressor cells (MDSC), both in vitro and in cancer patients. We found that both DC and MDSCs suppress NK cell responses in patients with cancer. STAT-3 phosphorylation status in DC determined NK cell responses. Furthermore, the crosstalk between DC and NK cells was regulated through production of LTA, IL-12, and TGF- β . We found that prostaglandin E2 (PGE2) converts healthy monocytes to MDSC-like cells. Similar to patient-derived MDSCs, PGE2-treated monocytes showed increased phosphorylation of p38MAPK/ERK and suppressed NK cell responses by the production of TGF- β .

In this thesis, new strategies to improve NK cell-based therapy have been suggested and new findings about the mechanisms of suppressing NK cells in the tumor microenvironment have been revealed.

LIST OF PUBLICATIONS

- I. **Sarhan D**, Wennerberg E, D'Arcy P, Gurajada D, Linder S, Lundqvist A. A novel inhibitor of proteasome deubiquitinating activity renders tumor cells sensitive to TRAIL-mediated apoptosis by natural killer cells and T cells. *Cancer Immunol Immunother.* 2013;62(8):1359-68
- II. **Sarhan D**, D'Arcy P, Wennerberg E, Lidén M, Hu J, Winqvist O, Rolny C, Lundqvist A. Activated monocytes augment TRAIL-mediated cytotoxicity by human NK cells through release of IFN- γ . *Eur J Immunol.* 2013; 43(1):249-57
- III. **Sarhan D**, Palma M, Mao M, Adamson L, Kiessling R, Mellstedt H, Österborg A and Lundqvist A. Regulation of NK cell responses by dendritic cells via lymphotoxin-alpha, interleukin-12, and tumor growth factor-beta; implications for development of DC vaccines against cancer. *Manuscript*
- IV. **Sarhan D***, Mao Y*, Steven A, Seliger B, Kiessling R and Lundqvist A. Myeloid-derived suppressor cells inhibit NK cell activity through prostaglandin-E2 regulated TGF- β production. *Manuscript*

*Contributed equally

RELATED PUBLICATIONS

Not included in this thesis

1. Wennerberg E, **Sarhan D**, Carlsten M, Kaminskyy VO, D'Arcy P, Zhivotovsky B, Childs R, Lundqvist A. Doxorubicin sensitizes human tumor cells to NK cell- and T-cell-mediated killing by augmented TRAIL receptor signaling. *Int J Cancer*. 2013; 133(7):1643-52
2. Kiessling R, Okita R, Mougiakakos D, Mao Y, **Sarhan D**, Wennerberg E, Seliger B, Lundqvist A, Mimura K, Kono K. Opposing consequences of signaling through EGF family members: Escape from CTLs could be a bait for NK cells. *Oncoimmunology*. 2012; 1(7):1200-1201; Review
3. Okita R, Mougiakakos D, Ando T, Mao Y, **Sarhan D**, Wennerberg E, Seliger B, Lundqvist A, Mimura K, Kiessling R. HER2/HER3 signaling regulates NK cell-mediated cytotoxicity via MHC class I chain-related molecule A and B expression in human breast cancer cell lines. *J Immunol*. 2012 1;188(5):2136-45

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cell
BDCA3	Blood Dendritic Cell Antigen 3
BAT3	B-associated transcript 3
BCL	B-cell lymphoma
cDC	Conventional/classical dendritic cell
CMP	Common myeloid progenitors
CLP	Common lymphoid progenitors
COX2	Cyclooxygenase-2
CCL	Chemokine (C-C motif) ligand
CTL	Cytotoxic T lymphocytes
CMV	Cytomegalovirus
CLL	Chronic lymphocytic leukemia
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CAR	Chimeric antigen receptor
DC	Dendritic cells
DNAM-1	DNAX Accessory Molecule-1
DR	Death receptor
DcR	Decoy receptor
ER	Endoplasmic reticulum
EGFR	Epidermal growth factor receptor
EP	Prostaglandin E2 receptor
FoxP3	Forkhead box P3
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Granulocyte/macrophage progenitors
grMDSC	Granulocytic myeloid-derived suppressor cells
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HA	Hemagglutinin
IL	Interleukin
IFN	Interferon
Ig	Immunoglobulin
LPS	Lipopolysaccharides
LC	Langerhans cells
KIR	Killer-cell immunoglobulin-like receptor
LAK	Lymphokine-activated killer cell
LTA	Lymphotoxin-alpha
Ly	Lymphocyte antigen
M-CSF	Macrophage colony-stimulating factor
mDC	Monocyte-derived dendritic cells
MHC I, II	Major histocompatibility complex class I or II
MPP	Multipotent progenitors

MDP	Macrophage-DC progenitors
MICA/B	Major histocompatibility complex class I-related chain A/B
MCA	3'-methylcholanthrene
MDSC	Myeloid-derived suppressor cells
Mac	Macrophages
moMDSC	Monocytic Myeloid-derived suppressor cells
mAB	Monoclonal antibodies
NK	Natural killer cells
NCR	Natural cytotoxic receptor
NKG2D	Natural killer group 2, member D
pDC	Plasmacytoid dendritic cells
PGE2	Prostaglandin E2
PD	Programmed cell death
PD-L1	Programmed cell death-ligand 1
PVR	Poliovirus receptor
PBMC	Peripheral blood mononuclear cell
ZA	Zoledronic acid
RAG	Recombination-activating gene
regDC	Regulatory dendritic cells
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription
TLR	Toll-like receptor
TCR	T cell receptor
Th	T helper cell
TNF	Tumor necrosis factor
TGF	Transforming growth factor
Treg	Regulatory T cells
TAP	Transporter associated with antigen processing
TRAIL	TNF-related apoptosis-inducing ligand
TAM	Tumor associated macrophages
TIL	Tumor infiltrating lymphocytes
ULBP	UL16-binding protein
VEGF	Vascular endothelial growth factor
WT	Wild type

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1 INTRODUCTION

The advantage of writing a thesis unlike writing a scientific paper is that for the first time as a scientist I can freely express myself without adhering to journal guidelines.

In this thesis I will describe my research findings but first give an introduction to relevant areas in the field of immunology to help understanding my work. For detailed information about basic immunology, text books such as Cellular and molecular immunology (1), and Janeway's Immunobiology (2), are recommended.

The introduction is divided into two parts where the first part describes important aspects of the immune system and the second illustrate the advances in basic and applied tumor immunology with a particular focus on natural killer (NK) cells. Though my work has mainly focused on human immunobiology in healthy individuals as well as in cancer patients, I have also used animal models to test our hypotheses.

With a new generation comes new ideas and what we know today is likely to be changed tomorrow. As a researcher in life sciences, I am blessed with the opportunity to be involved in such process. In this thesis, you will notice novel findings, which have added new considerations to the field of tumor immunology.

1.1 THE IMMUNE SYSTEM

Billions of microorganisms that may cause different diseases surround human body; therefore, mechanisms of defense are needed. Immunity, from Latin *immunitas* and means status of protection was first defined in the 1880s as a result of Jenner's, Pasteur's and Koch's work where they showed that entry of specific microorganisms may cause a certain disease and such illness can be prevented by vaccines that are extracted from the same pathogen (3). In 1890s, the natural and the artificial immunity were described as the following; Natural immunity is the resistance to pathogens from the first encounter, whereas the artificial immunity is to resist diseases caused by microorganisms that were hosted by the body in an earlier time point (4). Today we know that the immune system consists of cellular immunity and soluble factors. It is further classified conventionally into innate- and adaptive-immunity. According to immunology text books, the innate immunity, also called native or natural immunity, is the first line defense against microbes. Cells of the innate immune system include phagocytes (monocytes, macrophages, and dendritic cells) and natural killer cells. Additionally, soluble factors such as cytokines (small proteins that work as messenger, regulators, and sometimes as killers) do also belong to the innate immunity (5). In contrast, adaptive immunity responds slower to primary infections compared with the innate immune system. However, cells of the adaptive immune system (T cells and B cells) are specific and have a more powerful response when encountering pathogens for the second time (1, 5). Soluble factors of the adaptive immunity are antibodies that are produced by mature B cells (6). A very important distinction between innate from adaptive immunity is that the adaptive immunity can develop memory and can respond much faster upon rechallenge with the same pathogen (2).

Among the innovative steps to immunize for different pathogens was taken in the 18th and 19th century by the English physician Benjamin Jesty who started his work of cowpox vaccine that even resulted in immunization against smallpox, more than 20 years before Edward Jenner (7, 8). The idea of Jesty was to inoculate material from a sick cow in healthy individuals starting with his family (9). On the other hand, the credit was given to Jenner due to his exceptional work to development vaccines and eradication of smallpox (10). At that time, the dairymaids were generally known to be protected from the

smallpox. Jenner was inoculating an 8-years old boy with material from fresh cowpox lesions from a dairymaid. About a week later the boy developed a mild fever. Two months later Jenner was inoculating the same boy with a matter from fresh lesions from smallpox. The boy developed no disease and Jenner concluded that he was protected (11). This was the first footstep towards immunizing small children from common diseases. In the line with these discoveries, Louis Pasteur in the 19th century, took the development of vaccines to an advanced level, where he immunized with killed pathogenic agents to prevent development of diseases (12). Later in the 19th century Paul Ehrlich a German Jewish scientist laid the foundation for antibodies development describing, a specific antigen can (antigen from antibody generator: is a foreign or “self” element that provoke immune responses) generate an antibody response (13).

The immune system is a complex network but very much regulated. The early discoveries led to today’s knowledge. Below, components of the immune system, relevant for this thesis are described.

1.1.1 Monocytes and Dendritic cells of the innate immunity

Monocytes are large leukocytes originate from the myeloid lineage. They have different functions and participate in many essential innate immunity responses including phagocytosis, and cytokines production (14, 15). Circulating monocytes differentiate to macrophages and dendritic cells when resident in the tissues (16). They are also divided to two subsets; the classical subset is expressing high CD14, the co-receptor for Toll-like receptor (TLR)-4 to recognize bacterial lipopolysaccharide (LPS). They display an antibody-dependent cell-mediated cytotoxicity (ADCC) (17). However, under certain conditions, they can also suppress activated lymphocytes (18, 19). The other subset of cells express low levels of CD14 and high level of HLA-DR, a good presenter of peptides derived from viruses and bacteria, and also produces high levels of interferon-alpha (20, 21). The majority of monocytes do not express FcγIII receptor (CD16), however about 10% of the blood circulating do. Those monocytes expand by cytokine treatment including; GM-CSF, M-CSF, and IL-3 and also during inflammatory

conditions like, coronary artery disease and chronic kidney disease (22-26). Monocytes display plasticity and can function as pro-inflammatory and anti-inflammatory when needed (27, 28). Pro-inflammatory monocytes can stimulate both memory T cells and NK cells in a cytokine-dependent manner during microbial invasion (29-31). In allergic reactions monocytes are recruited to the site of inflammation to dampen such reaction as an anti-inflammatory component (32).

Dendritic cells (DC) originate from either myeloid or lymphoid progenitors (33-35) (**Figure 1**).

In mice, DC are divided into classical DC (cDC), plasmacytoid DC (pDC), and Langerhans cells (LC). Those subsets are divided further to unique groups of DC according to their surface antigen expression and location. The cDC are found in lymphoid tissues with either $CD8^+CD11c^+CD11b^-$ or $CD8^-CD11c^+CD11b^+$ phenotype (36, 37). In non-lymphoid tissues, are they either $CD103^+CD11b^-$, $CD103^-CD11b^+$, or $CD103^+CD11b^+$ in intestine (38, 39). pDC are blood and spleen residents characterized by expressing low levels of major histocompatibility complex (MHC) class II and CD11c but produce high levels of type I IFN (40). LC populate the skin and express high level of langerin and are $CD11b^+F4/80^+$ (41).

In humans it is more complicated to distinguish DC subpopulations where many surface markers are shared with other hematopoietic cells such as monocytes and macrophages (42), DC are typically divided into conventional/classical DC (cDC), plasmacytoid DC (pDC), Langerhans cells, and monocyte-derived DC (mDC) (43, 44). cDC are found in the blood circulation under both steady and inflammatory conditions and express high level of CD11c, CD1b/c and BDCA3. They are highly migratory and generally short-lived in the blood. (43, 45). In contrast, mDC were primarily included in the non-conventional DC since they do not differentiate in normal conditions but only during inflammatory conditions (46, 47). Yet, recent studies have suggested that mDC exist in intestines, and muscles in a steady state (48, 49). pDC are long-lived cells usually found in the circulation or lymphoid tissues. They express low levels of MHC-II and CD11c with high

ability to produce IFN- α when stimulated. LC, sometimes referred to “skin DC”, mainly reside in the epidermis (50, 51).

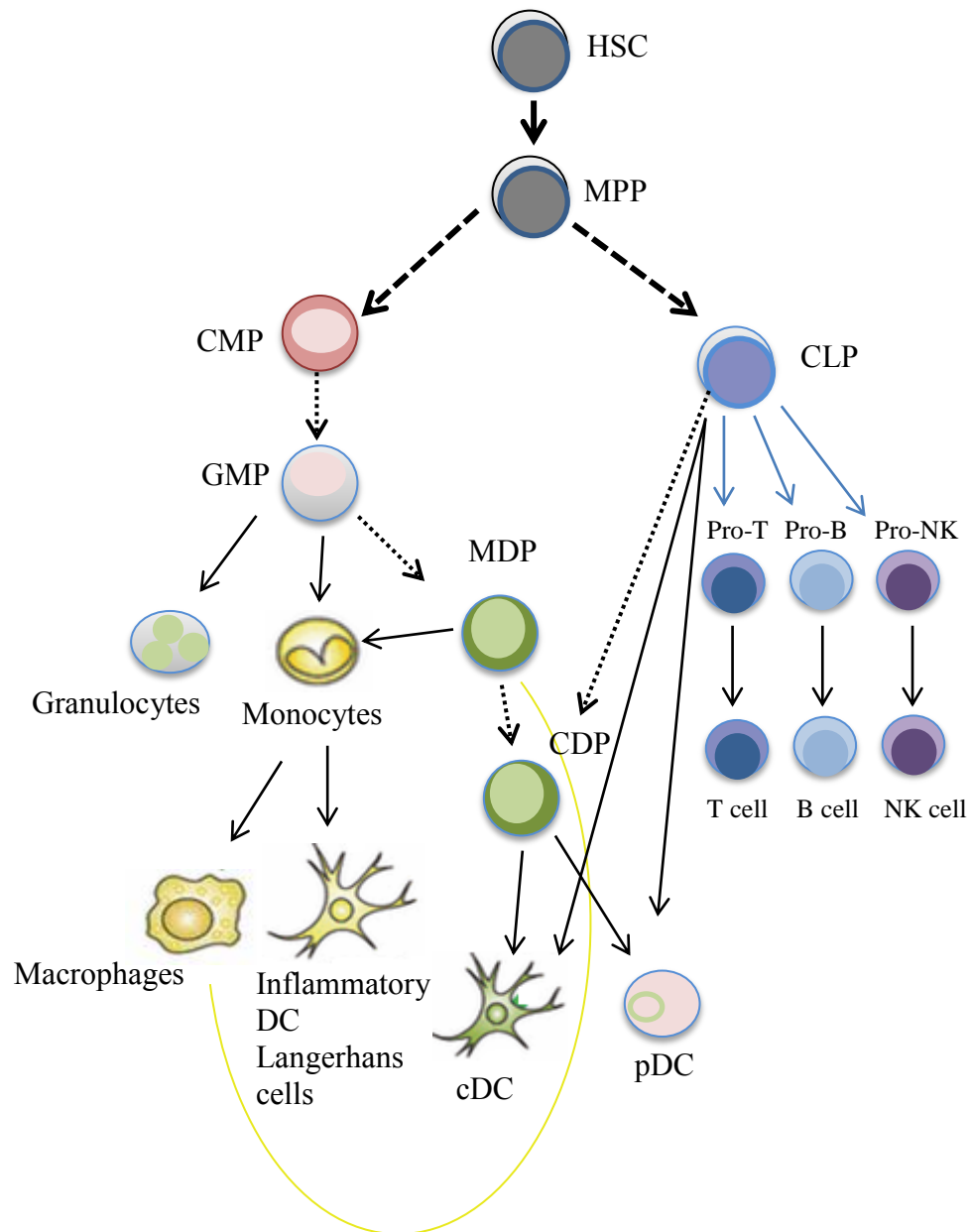


Figure 1: Hematopoietic tree for dendritic cell development. Hematopoietic stem cells (HSC) in the bone marrow give rise to multipotent progenitors (MPP). Further downstream, lineage differentiation splits to common myeloid progenitors (CMPs), or common lymphoid progenitors (CLPs). CMPs then further differentiate into granulocyte/macrophage progenitors (GMPs).

Macrophage-DC progenitors (MDPs) give rise to monocytes and conventional DCs (cDCs), and plasmacytoid DCs (pDCs). MDPs-derived monocytes can further differentiate into inflammatory DCs and Langerhans cells. CLPs can give rise to both common DC progenitor (CDP) and pDC.

DC are typically referred to as professional antigen-presenting cells. To activate naïve T cells, DC deliver three typical activation signals, stimulatory signal 1 presenting of antigens to T cell receptor (TCR), co-stimulatory signal 2 when binding to CD28 co-stimulatory receptor, and polarization signal 3 which is mediated by cytokine or membrane-bound stimulation like IL-12 or CCL2 (52).

1.1.2 Cytokines

Cytokines are small proteins involved in diverse biological responses including; proliferation, cell growth, cell differentiation, inflammation, defense against microbes, etc. (1, 53-56). Cytokines are produced by many different cell types like leukocytes and some epithelial and endothelial cells. They act by binding to their receptors which are expressed selectively on specific subsets of cells. Failure to produce cytokines or dysregulation of their function can lead to different diseases including autoimmune diseases and cancer (57-59). There are different cytokine families including the interleukins (IL), the tumor necrosis factors (TNF), and the interferons. Among the first described ILs is IL-2, which is essential for proliferation, survival and enhancement of killing of T- and NK-cells (60-62). Together with other cytokines IL-2, IL-15, IL-12, IL-6, TNF- α , lymphotoxin-alpha (LTA), and IFN- γ belong to a group called proinflammatory cytokines due to their ability to induce inflammation (63, 64). In contrast, cytokines like IL-10 and TGF- β are anti-inflammatory cytokines due to their involvement of suppressing immune responses (65-67).

1.1.3 T cells of the adaptive immunity

Optimal defense against different microorganisms needs the action of the adaptive immunity. Such action can be derived by thymus-derived (T) cells, using antigen-specific receptors to mediate effector responses in two steps. First, when T cells are primed by antigen-presenting cells (monocytes, macrophages, DC, and B cells) in the secondary lymph node, they become activated, differentiate to different subsets depending on the received signal and proliferate. Primed T cells then migrate to the site of infection in response to chemokines usually produced by innate immune cells that are already presented at the site. T cells of the adaptive immunity are typically classified as CD4 Th1 inflammatory cells that activate macrophages, CD4 Th 2 help to generate antibody responses, regulatory T cells negatively regulate effector cells, and importantly CD8 cytotoxic cells that are able to mediate direct killing of the target (1, 6) (**Figure 2**).

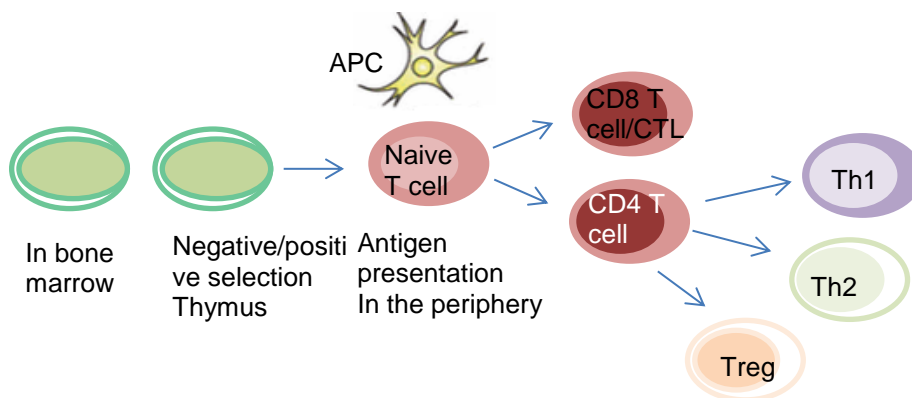


Figure 2: Differentiation of T cells into different subtypes.

1.1.4 Antigen processing and presentation

The classical way to present antigens is on the molecular complex of the MHC class I or II. MHC molecules present either endogenous (intracellular) or exogenous (extracellular) proteins (68). The process of antigen presenting begins with proteolytic degradation of the aimed protein. The process of degrading proteins is a very important biological act in the cell to dispose of accumulated and destroyed proteins (69, 70). The proteasome system is utilized for such purpose. The proteasome is a multi-subunit complex consist of 20S core unit and different regulatory subunits that bind to the 20S, like the 26S and 19S to modify and select degradation (71). All proteasomes have similar structure consisting of three subunits yet the immunoproteasome containing the 20S core unit and one regulatory unit such as 19S or 11S (72) (**Figure 3**). The immunoproteasome is used in the MHC class I restricted antigen presenting (73, 74).

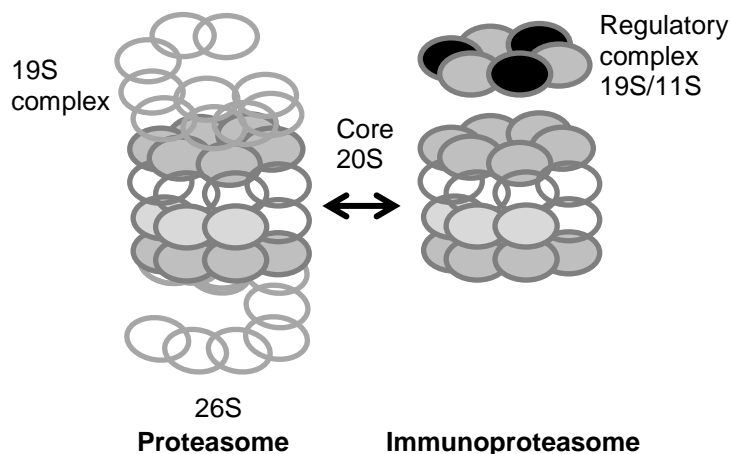


Figure 3: Proteasome and immunoproteasome multi-subunit structure consisting of core subunit and regulatory subunits.

1.1.4.1 *Endogenous antigen presenting- MHC I*

Cytotoxic T cells ($CD8^+$) are activated by MHC class I molecule. Such molecule is expressed on all nucleated cells this means all cells can present antigens to $CD8^+$ T cells. The MHC I molecule is presenting cytosolic antigens that can be products of viruses, other intracellular microbes, or tumor cells. Other source of antigens is phagosomes (vesicles) that carry microbes or products of pathogens which internalize to the cell cytosol and be processed as the other cytosolic antigens. Following processing, antigens are transported to the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP), packed on an MHC I molecule and via the Golgi transported with exocytic vesicles to the cell surface (1) (**Figure 4**).

1.1.4.2 *Exogenous antigen presenting- MHC II*

MHC class II molecules are expressed on few cell types, as such antigen presenting cells. On MHC II molecules are the exogenous antigens presented to the $CD4^+$ T cells. On the other hand a process called cross-presentation enable exogenous antigens to be presented on the MHC I. Unlike the endogenous presenting, the loading of MHC II is occurring outside the ER where only MHC II is processed following a loading step inside the fused endocytic and exocytic vesicles in the cytosol and finally presented on the cell surface (1) (**Figure 4**).

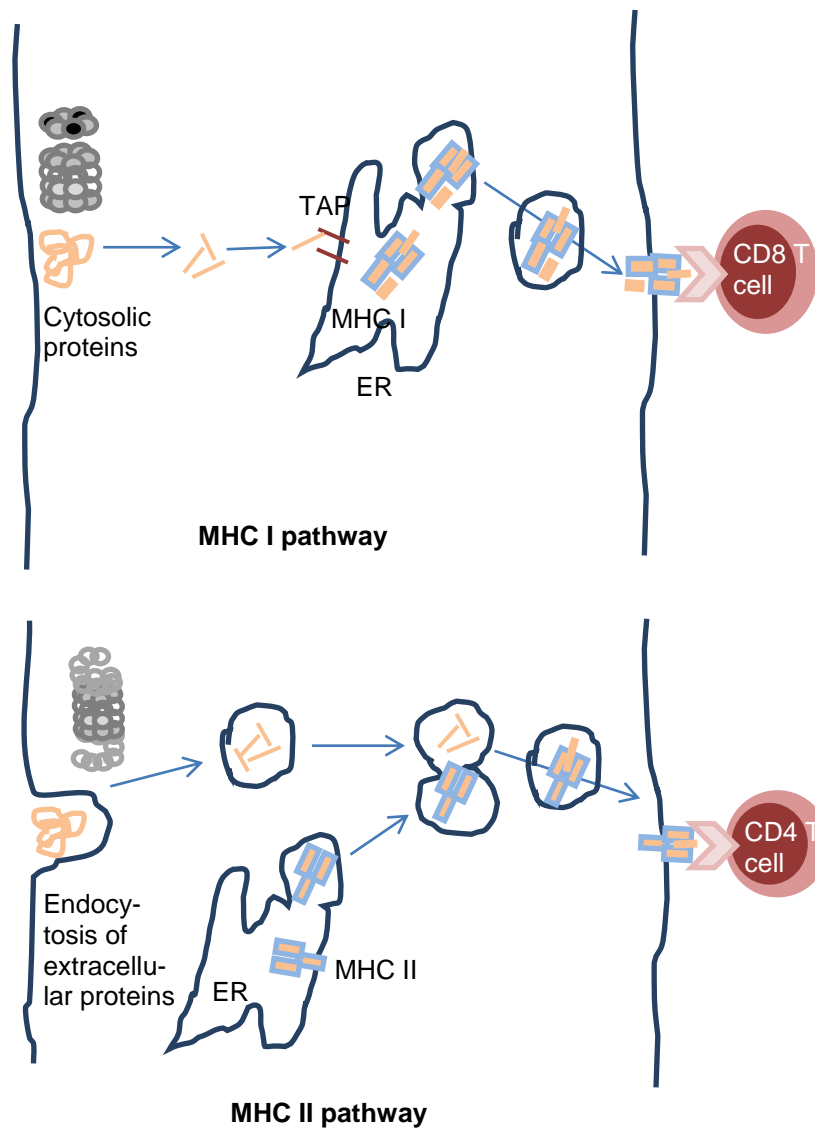


Figure 4: Classical antigen presentation. Antigens are either presented by MHC class I or II molecules that requires different processing and loading of the antigens.

1.1.5 NK cells

1.1.5.1 *NK cell identification*

In the mid-70s, the term “natural” was used based on the functional properties of NK cells where they recognize and lyse certain tumor cells without requirement of prior immunization (75, 76).

NK cells consist of 5-15% of the blood circulating lymphocytes and described as large granular lymphocytes of the innate system due to their inability to rearrange their receptors from their germline configuration (77, 78). They are identified by the expression of CD56 and lack of CD3. CD56 (NCAM) is found on human NK cells but not on those of murine origin (79). However, studies have suggested that a natural cytotoxic receptor NKp46 can be used as an identification marker of NK cells across different species (80, 81). In conflict, a subset of human NK cells lack or express very low NKp46 which results in difficulty to identify NK cells therefore, they have to be determined by the lack of other lineage markers (82).

1.1.5.2 *NK cell development*

The localization where NK cell development occurs in humans has been argued. Though they originate from bone marrow CD34⁺ hematopoietic progenitor cells, their differentiation into mature cells are not thought to take place in the bone marrow (83). Instead, NK cells are believed to mature in the lymphoid tissues due to the lack of immature and intermediate matured NK cells in the bone marrow. On the other hand, in vitro studies have shown that CD34⁺ cells are differentiated to NK cells by cytokines which are normally produced by immune cells located in the bone marrow (82, 84, 85).

Human NK cells are divided into two subtypes based on their expression level of surface CD56. CD56^{bright} (high level of CD56) are marked as immunoregulatory cells with remarkable cytokine production capacity and resident of the lymphoid tissues, while CD56^{dim} (low expression of CD56) constitute 90% of NK cell population are circulating in the blood. Unlike

CD56^{bright}, CD56^{dim} express high levels of CD16 and have potent cytotoxic functions (**Figure 5**) (86, 87). Cells expressing CD16 are able to kill target cells via antibody-dependent cell-mediated cytotoxicity (ADCC) (88).

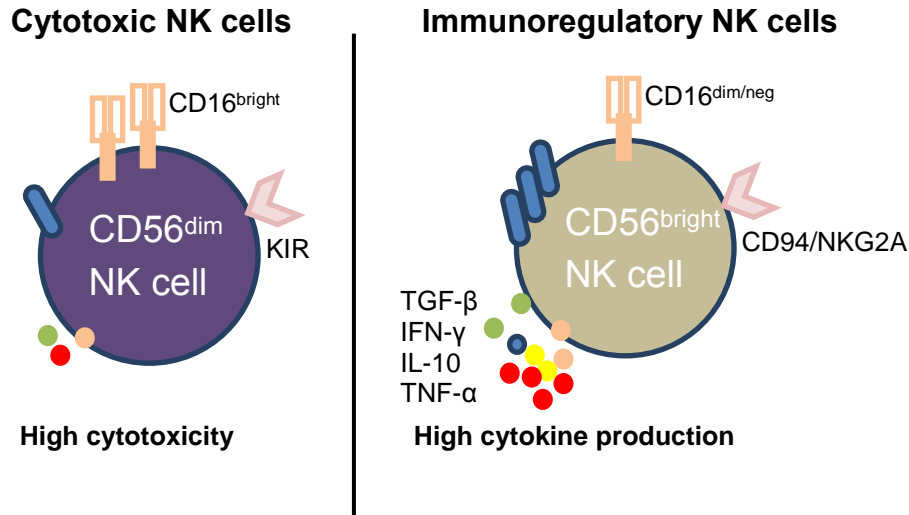


Figure 5: Different NK cell subsets classified depending on their expression levels of CD56 and CD16 expression. CD56^{dim} is the cytotoxic NK cell and CD56^{bright} is the immunoregulatory NK cell.

1.1.5.3 *Inhibitory receptors*

NK-cell function is regulated through a balance of inhibitory and activating receptors. The final response is predicted by the strength of binding or lack of binding of the inhibitory receptors. The largest group of inhibitory receptors are the killer immunoglobulin-like receptors (KIRs) that bind to the MHC class I complex (89). In the beginning of the 80s, Kärre and co-workers observed that the murine tumor cell line YAC-1, expressed low levels of MHC class I molecules, were sensitive to killing by NK cells (90). These observations later led to the formulation of the “missing-self” hypothesis (**Figure 6**) (91).

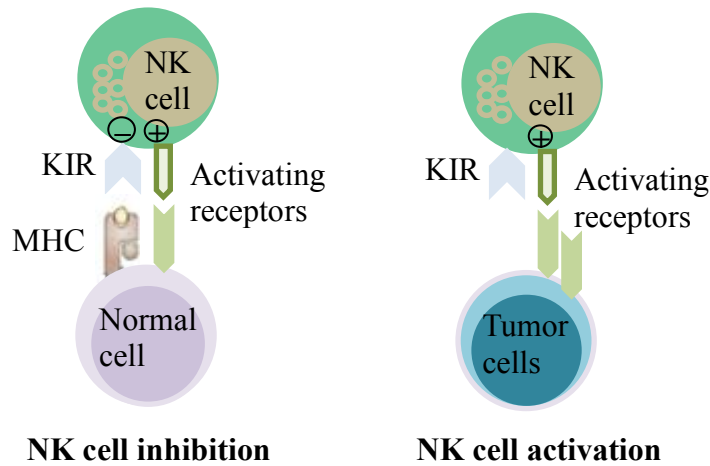


Figure 6: The “missing self” model. NK cell inhibition mediated by KIR ligation of MHC I molecule in normal cells. When cells are transformed and lose their MHC I expression the inhibition of NK cells has disappeared and they are able to kill their target.

In the 1992 in the laboratory of Yokoyama, the first inhibitory receptor Ly49 expressed on murine NK cells was identified (92). Later, in the beginning of the 90s, Moretta and colleagues were first to discover the human KIRs of the NK cells (93-95). Another inhibitory receptor expressed by NK cells is the CD94/NKG2-inhibitory receptor (96).

1.1.5.4 *Activating receptors*

In addition to inhibitory receptors, NK cells also express activation receptors. One of the most studied receptor is FCR γ III (CD16), which is expressed on the majority of the NK cells and binds the constant region (Fc) of IgG (86, 97). CD16 is the only receptor that activates NK cells by itself (98, 99). Among other

important activation receptors are, NKG2D which ligate the stress induced molecules MICA/B or ULBP-proteins, and natural cytotoxic receptors (NCRs, NKp30, NKp46, and NKp44) (100-105). The ligands to the NCRs are not very well studied. What is known today, the two described NKp30 ligands BAT3 and B7-H6 and hemagglutinin (HA) a viral protein that ligate NKp44 and NKp46 (106-110). Add to the list, the activation co-receptors that are involved in the regulation of NK cell reactions to the target including; DNAM-1 and NKp80 (**Figure 7**) (111, 112). These activating receptors play an important role in stimulating NK cells in different pathogenic and in cell transformation conditions.

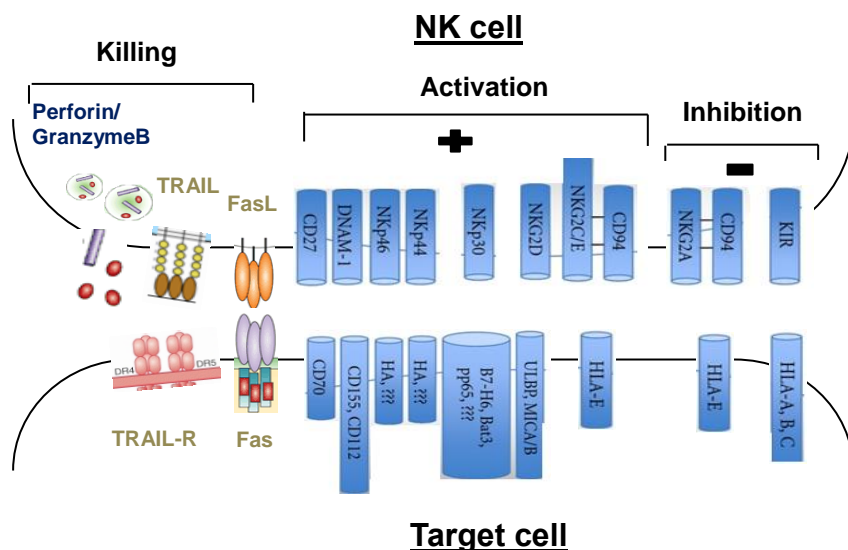


Figure 7: NK cell receptors. NK cells interact with their target by integrating inhibitory and activation receptors.

As such action, ADCC has been widely recognized as a process to target virus-infected cells as well as tumor cells (113, 114). The expression of different activation or inhibitory isoforms of NKp30 in gastrointestinal sarcoma patients

predicts the clinical outcomes (115). In the line of the diversity of NK cell responses is the ligation of NKG2D by MICA/B or ULBP proteins has been shown to induce cytotoxic activity against hepatoma cells (116). The co-receptors of the NK cells have been revealed to be important for fighting cytomegalovirus-infected cells and might play a role in the tumors where NK cells display an altered phenotype (117, 118).

Importantly, the above mentioned receptors except CD16, need to cooperate with each other to stimulate NK cell action (119). For instance, activating NKp46 alone is not sufficient to activate NK cell degranulation; however co-activating DNAM-1, NKG2D, or 2B4 was required for NK cells to degranulate (99).

1.1.5.5 *Regulation of NK cell receptors*

During the development of NK cells, receptor expression is highly regulated to avoid autoimmunity or hypoactivation. Intensive investigations of how the KIR family expression is regulated have shown that NK cells normally in healthy individuals express at least one KIR. However, there are a heterogenic repertoire expressed among the cells (120). There are few ideas of how the expression of NK cell receptors is regulated. One of those is NK cell education or licensing, such idea mimics the theory behind TCR repertoire except that NK cell receptors are not rearrangeable. NK cells undergo an educational process by binding diverse MHC I molecules to their KIRs. Studies in mouse-models have shown that NK cell licensing is associated with a correlation of self-MHC class I expression and expression of an inhibitory receptor for a self-MHC class I on NK cells (121). Such interaction is needed for a fully responsive NK cells and for self-tolerance (122).

In the line of regulating receptors, a very strong regulator of the activation receptors is the receiving of proinflammatory respectively anti-inflammatory cytokines including; IL-12, IL-15, TGF- β , and IL-10 (123-126). Although, there

are accumulating studies examining the regulation of NK cell receptor expression in different human disorders, we still lack the complete understanding of such processes especially in cancer patients.

1.1.5.6 *NK cell cytotoxic action*

Following activation or lack of inhibition, NK cells produce pro-inflammatory cytokines like IFN- γ and TNF- α (127-130). In response to target cells, NK cells integrate by their adherent molecules like LFA-1 resulting in Ca⁺ release which further polarize cells, and degranulate the perforin containing lytic granules (131, 132). Other direct killing mechanism involves ligation of death receptors. Death ligands expressed on the surface of NK cells, including TNF-related apoptosis-inducing ligand (TRAIL) and Fas-ligand (FasL) bind to their cognate receptors expressed on target cells. While the FasL binds to its only receptor Fas, TRAIL is binding to four known membrane-bound receptors and one soluble receptor (**Figure 8**). TRAIL-receptors DR4 and DR5 acting in two steps initiated by binding their extracellular domain to TRAIL which results in transforming the signal to the intracellular domain, such signal recruit and activate the caspase in turn initiate the apoptotic signal-transduction pathway (133). Normal cells usually are discriminated from transformed cells by expressing the other two membrane-bound TRAIL-receptors called decoy receptors DcR1 and DcR2 which either lack or have no functional intracellular death-domain (134-137).

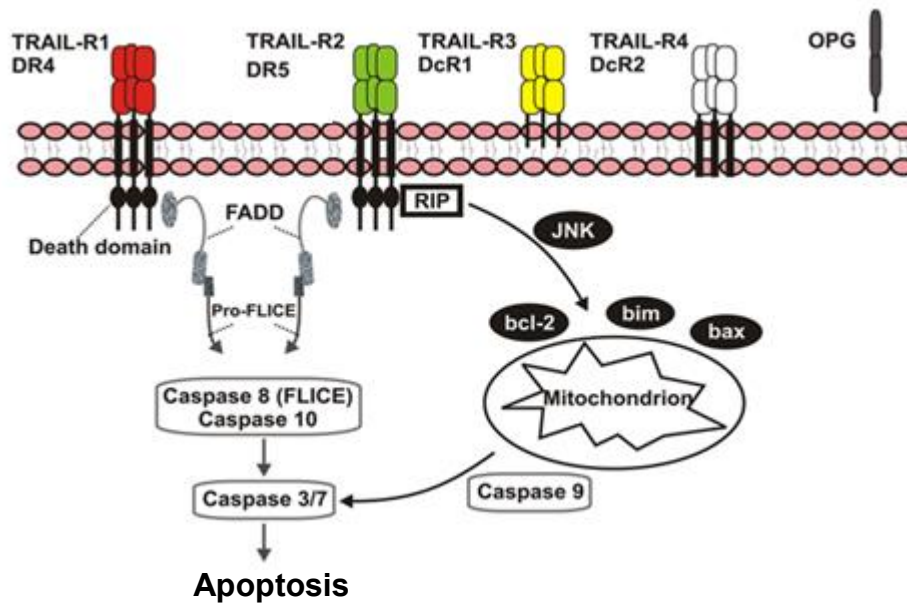


Figure 8: Human TRAIL-R expression. Different TRAIL-R extracellular and intracellular domain expression in human normal cells. The figure is modified form *Front Biosci.* 2007 May 1;12:2912-21.

1.1.5.7 Memory NK cells

NK cells are traditionally described as short-lived innate lymphocytes with a limited proliferative capacity and the lack of antigen specificity. However, recent studies have challenged this dogma by demonstrating unexpected observations that NK cells display adaptive immune characters. O'Leary et al. demonstrated that a subpopulation of liver-resident NK cells is capable of mediating specific hapten-induced responses in mice lacking T and B cells in terms of a second challenge (138). CMV infection may result in the generation of memory NK cells in mouse models (139). The NK cell memory is still a new concept that has been evaluated mostly in virus-infection models (140). Further investigations in other diseases models are required.

NK cell respond differently in healthy and in disease conditions. In the next session the NK cell responses in the tumor microenvironment are discussed.

1.2 TUMOR IMMUNOLOGY

The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors. These include; sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (141). Progress in tumor biology research has added two new hallmarks; reprogramming of energy metabolism and evading immune destruction (142).

1.2.1 Immune surveillance

The role of the immune system in controlling transformation cells was very controversial in the 1909 when Ehrlich first suggested the idea (143). Later during 1950s Burnet revised the topic “natural” protection against tumors. Burnet believed that tumor cell-specific antigens could break the immune tolerance and provoke an effective immunologic response that results in eliminating carcinogenesis (144, 145). Eventually, the idea was accepted in the 1990s when the approach of transgenic mouse-models led to the validation of tumor immune surveillance both in chemical induced as well as spontaneous tumors. At that time the role of effector cells as T-, B-, and NK-cells, and interferons was confirmed to be critical (146-148).

Another evidence for immune surveillance was emerged in transgenic mice lacking the gene RAG that is essential for rearrangement of immunoglobulin and T cell receptors were subcutaneously injected with the chemical carcinogen 3'-methylcholanthrene (MCA). 60% of the mice developed sarcomas compared with 19% of the WT mice (148). Additional evidence support the immune surveillance is, immunosuppressed transplant recipients that have an increased risk of

developing cancers (149, 150). Finally, in the last decade several studies from cancer patients have shown that high numbers of infiltrated lymphocytes, especially T cells correlate with good prognosis (151-154).

1.2.2 Immune escape

There are several factors that inhibit or generate the development of cancer. Such factors can be mediated by the transformed cells (tumor-cell-intrinsic) or by the surrounding (tumor-cell-extrinsic). The development of cancer involving three important steps of the cancer immunoediting namely, the “3 Es” model; elimination, equilibrium, and escape (**Figure 9**).

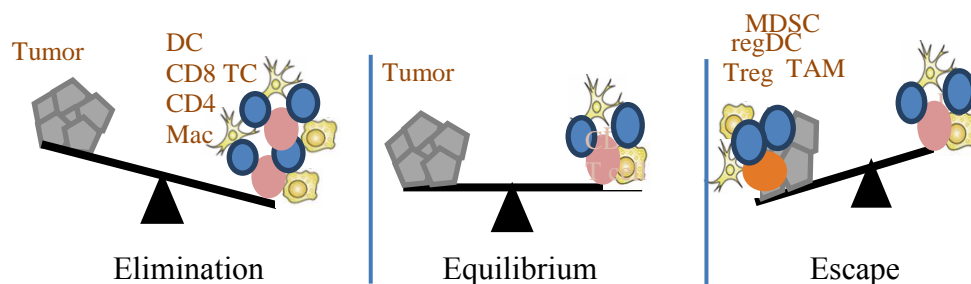


Figure 9: Tumor immunoediting. The balance of tumor immunosuppression and immune cell action.

The elimination is a process of protection from uncontrolled growth that results in eradicating the damaged cells by the immune system i.e. immune surveillance (155-157). Proceeding to the next step “equilibrium”, when tumor cells resist the immune effector cells under long time, consequently they become less immunogenic, emerging immune selection of tumor cells where more resistant

cells survive (158). The final step is when the immune system has no or very limited control on the tumor mass, here the tumor immune “escape” has been reached (159).

The immune escape by tumors is recognized and can be divided into three categories; loss of recognition, absence of susceptibility, and induction of immune suppression (160). One of the most known examples of loss of recognitions is the failure to present tumor specific antigens where T cells are no longer able to recognize those (161). Furthermore, tumor cells usually, turn off signals, down-regulate, or shed receptors or ligands that are necessary to induce killing by effector cells which results in tumor lack of susceptibility (162-164).

1.2.3 Tumor microenvironment

The tumor microenvironment is known to consist of a heterogeneous population of cells and secreted factors including; not only the tumor cells, but the immune cells and cytokines. In this session, selected cell populations and factors involved in the tumor microenvironment are described.

1.2.3.1 *Intrinsic factors: Membrane bound proteins*

During immune editing, tumor cells lose or over-express different proteins that might be very important for immune recognition or suppression. HLA-molecules are of fundamental importance for T cell priming and activation. Failure to present antigen has been observed both in murine tumor models and in cancer patients. Such deletion of antigens is mediated by mutations in the antigen-presenting machinery or due to HLA complete loss, or even loss of the antigen expression (161, 165, 166). Tumor cells that have lost the expression of MHC class I are insensitive to killing by T cells. Instead, such tumor cells are sensitive to killing by NK cells. However, tumor cells can resist NK cell killing via down-regulation or shedding of death receptor Fas, and stress ligands for NKG2D, or down-modulating TRAIL-receptor-mediated apoptosis by upregulating cFLIP (167-169).

Recently, much attention has been paid to the programmed death ligands 1 and 2 (PD-L1/PD-L2) that are expressed on wide range of tumors. They interact with the receptor PD-1 that is found on activated immune cells to prevent hyperactivity (170-174). Upon interaction with tumor cells both NK cell and T cell antitumor activity is impaired and clinically correlated with poor prognosis (175-177). Other similar molecules that are overexpressed and inhibit T cell and NK cell functions by integrating with tumors are CTLA-4, HLA-G, and HLA-E. Note that these molecules act early to mediate immune-suppression (178-181).

1.2.3.2 *Intrinsic factors: tumor-induced transcriptions factors*

Another type of immune-suppression mediated by tumor cells is the high activity of certain transcriptional factors namely, signal transducer and activator of transcription (STAT)3 that is constitutively phosphorylated in many tumors (182, 183). STAT3 activity in tumors has been associated with inhibited immune surveillance by CD8⁺ T cells and Th1 cells (184, 185). The STAT3 activity of tumors induces production of suppressive cytokines as IL-10 and TGF- β (186, 187).

1.2.3.3 *Secreted factors in the tumor microenvironment*

The factors secreted in different cancers might be different in the type and the amount, however “master” regulators seems to be commonly produced. Among these suppressive factors are TGF- β (188), IL-10 (189), IL-6 (190), GM-CSF (191) and inflammatory mediators like Cyclooxygenase (COX-2) and prostaglandin E2 (PGE2) (192). The suppressive cytokines as TGF- β within the microenvironment block for instance the differentiation and maturation of dendritic cells which results in inhibited cross-presentation of tumor-antigens to the T cells (193). Another potent inhibitor is IL-10 where it has a direct effect on the production of TNF- α and IFN- γ by NK cells and T cells. It also inhibits the cytotoxic effect of macrophages and their ability to produce IL-12 (194, 195). The

immune-modulators mentioned above contribute to a cascade of inflammatory responses that promote cancer progression by inhibiting DC, skewing cytokine production, enhancing angiogenesis, and inhibiting apoptosis (196-199). Collectively, tumor progression is frequently associated with severe impaired host innate and adaptive immune responses.

The immune-suppressive environment of tumors includes complicated mechanisms and involvement of many immune cells and factors. Therefore it is impossible to include all of them in this thesis. In the next session the role of NK cells and their interaction with tumor cells and selective immune-suppressive cells is highlighted.

1.2.4 Immune suppression of NK cells

In order to understand NK cell responses against cancer we need to know more about how NK cells interact with not only tumor cells, but how they perform in the tumor microenvironment.

1.2.4.1 *Immune-suppressive compartments: Regulatory T cells*

One of the most studied immune-suppressive cell type associated with tumor progression is regulatory T cells (Treg). They are characterized by their expression of CD4, CD25, CD127 ($CD4^+CD25^+CD127^{low/neg}$) as well as the transcription factor forkhead box P3 (FoxP3) (200). The expansion of Treg is promoted in different cancers and their accumulation correlates with impaired immune cell-function and poor prognosis (201-206). NK cells are suppressed by Treg in a cell-contact dependent manner where membrane-bound TGF- β is utilized to attenuate their cytotoxicity (207). Importantly, Treg express the high affinity IL-2 receptor- α (IL-2R α) and need IL-2 for their full-function. They consume IL-2 that is produced by other cells suggesting the idea of IL-2 deprivation as a mechanism of suppression of T cells and NK cells (208, 209).

1.2.4.2 Immune-suppressive compartments: Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of precursors of granulocytes, macrophages, and immature DC with suppressive activity (210). Recently, myeloid-derived suppressor cells (MDSCs) have been proposed as key immunoregulators in various solid and hematologic malignancies (211, 212). MDSC are divided into two groups the granulocytic (grMDSCs) and the monocytic MDSCs (moMDSCs) (213). In mice grMDSCs are characterized by expressing CD11b, Gr-1, Ly6G, and low Ly6C and moMDSC by CD11b, Gr-1, high Ly6C, and lack of Ly6C (213). In humans, distinct phenotypes of MDSCs are associated with different types of cancers (214-218). Their suppressive function is mediated by different mechanisms however mostly through suppressive cytokines including IL-10 and TGF- β or by depleting their surrounding by arginine or by production of reactive oxygen species (ROS) (**Figure 10**) (215, 219-222). Recent studies have aimed to investigate the induction mechanism of MDSCs and how they suppress T cells in vitro (223-225). Despite what is identified about the interaction between T cells and MDSCs, much less is known about how NK cell responses are influenced by these cells.

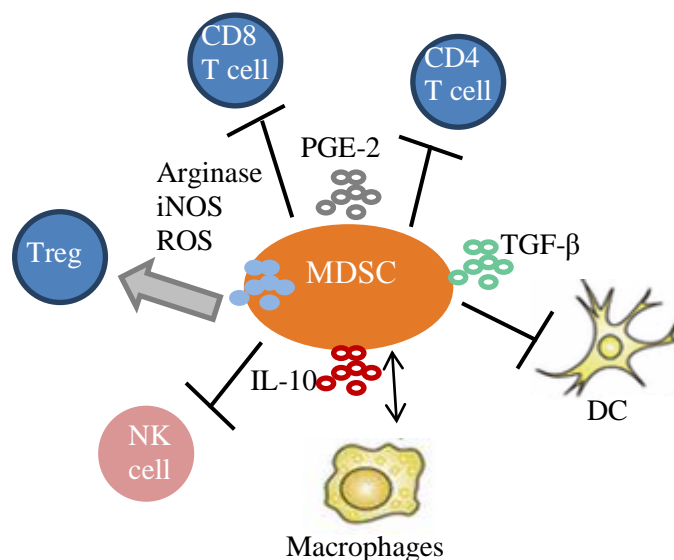


Figure 10: MDSC suppression. MDSCs apply different suppression mechanism on effector cells like NK cells, CD4 and CD8 T cells and also induce differentiation of other suppressive cells like Treg and TAM. The figure is a modification from *J Immunol.* 2009 Apr 15;182(8):4499-506.

1.2.4.3 *Immune-suppressive compartments: Tumor-associated macrophages*

Macrophages are the dominant myeloid-derived population that is found in the tumor microenvironment. Tumor-associated macrophages (TAMs) are suggested to actively promote tumor growth and metastasis (226, 227). TAMs are characterized as a population of multiple distinct pro (M2)- and anti (M1)-tumoral subpopulations. The immune suppressive mechanisms applied by TAMs in the tumor microenvironment can be recruitment of Treg, direct and indirect inhibition of T cells, and production of IL-10 (228-230).

1.2.4.4 *Immune-suppressive compartments: Immune-regulatory Dendritic cells*

Recent studies of less understood cell population, suggest pro-tumorigenic myeloid-derived immune regulatory cells namely, immune-regulatory Dendritic cells (regDC). They are derived from cDC and their infiltration in the tumors correlates with poor clinical outcome (231, 232). In a pre-clinical study of lung cancer, shows accumulation of regDC support tumor growth and suppress anti-tumor activity (233).

1.2.5 **Immunotherapy**

Immunotherapy aims to strengthen the immune system to eliminate cancer. Immunotherapy can be divided into passive where anti-tumor effectors are provided (antibody therapy, cell therapy) and active specific (vaccines) or active non-specific therapy (cytokines) where patients own immune system is stimulated (listed below).

Active immunotherapy

1. The aim of tumor vaccines is to induce immune recognition of the tumor cells or to boost the already existing anti-tumor immune response. However, an important concern in such type of therapy is the low immunogenicity of tumors and already reduction of effective immune compartments. Such vaccines includes; Cell based (whole-tumor cell vaccine, DC-vaccine), or

tumor component based (DNA vaccine, antigen peptide vaccine, and exosome-based vaccine) (234-237).

2. Cytokines are commonly used in immunotherapy for the purpose of stimulating the immune responses like differentiating, proliferating and activation of effector cells, and recruitment of APC. Such cytokines are IL-2, IFN- α , and GM-CSF, however these therapies have high toxicity rates due to their systematic administration (238).

Passive immunotherapy

1. Antibody-based therapy is one of the earliest strategies for cancer immunotherapy. Monoclonal antibodies (mAB) can directly kill the targeted tumor cells, stimulate component of the immune system (ADCC), or interfere with the inhibitory signaling pathways applied by the tumor cells. These mAB including; anti-CD20 (Rituximab), anti-HER2 (Trastuzumab), anti-VEGF (Bevacizumab), anti-EGFR (Cetuximab), anti-CTLA-4 (Ipilimumab) (239). One major concern with long-term antibody-based therapy is development of resistance (240).
2. Immune hyporesponsiveness is a major fact in the tumor microenvironment which initiated the idea about adoptive cell therapies with ex-vivo activated immune cells. Tumor infiltrating lymphocytes (TILs) have been shown to be reactive to host tumor-specific antigens, however inhibited in the tumor mass. Numerous studies have investigated the ability of expanding such cells ex-vivo and transfer them back to the patients. Such treatment has shown remarkable success in melanoma patients (241). Other T cell therapies include genetic modifications, specific-TCR transduced T cells and chimeric antigen receptor (CAR)-expressing T cells (242, 243).

As mentioned above, the tumor microenvironment plays a significant role in suppressing the immune response against cancer. Therefore, therapies aimed at

targeting immunosuppressive cell populations are emerging. Below additional immunotherapy targets are described.

1.2.5.1 *Targeting Treg*

The therapeutic targeting of Treg has achieved progress with limited success. Several therapeutic agents have been suggested to reduce Treg numbers; however those agents do not target Treg specifically but also target effector cell function. Such therapies target Treg proliferation (244, 245), impair their function by the TLR-agonists (246). Recently, the use of mAB CTLA-4 suggested as a target for regulating Treg in cancer patients. However, pre-clinical and clinical studies show an accumulation Treg though an increased anti-tumor activity by effector T cells, suggesting the indirect effect of CTLA-4 treatment (247, 248).

1.2.5.2 *Targeting MDSC*

MDSCs do not express specific identification markers which make them difficult to selectively deplete. Instead, investigators have explored to deplete immunosuppressive factors produced by MDSCs. Therapeutic preclinical examinations of different strategies are suggested including blocking of MDSC differentiation, expansion, and their suppressive function (249). Amiloride is used in patients with hypertension though treating tumor-bearing mice with amiloride decreased the STAT3-dependent MDSC suppressive activity by inhibiting tumor exosome formation (250). Sunitinib, a tyrosine kinase inhibitor and target many growth factors like M-CSF and VEGF-receptors. Administration of Sunitinib in tumor-bearing animals targeted the expansion of MDSC and enhanced Th1 IFN- γ expression (251). PGE2 block the differentiation of APC from the bone marrow and induce MDSCs (252). COX-2 inhibitors like celecoxib and Acetylsalicylic acid (Aspirin) reduce the systematic level of PGE2 consequently, reduced the MDSC production of ROS and arginase as well as the chemoattractant of MDSCs CCL2 (253-255).

1.2.5.3 *Blocking immunological checkpoints*

The aim of targeting immunological checkpoints is to accumulate the positive effect of the effector cells and eliminate the suppression effect of the suppressor cells like Treg and MDSCs in the tumors. Immunotherapeutic strategies were directed primarily on enhancing immune effector functions, as such CTLA-4 blockade by mAB (Ipilimumab). Ipilimumab has been now used in several clinical studies in cancer patients with good objective responses and stable disease (256-259). Another inhibitory member of the checkpoint proteins is the PD-1:PD-L1 axis considering promise ability in rescue exhausted CD8⁺ T cells in murine models (259, 260) and prolonged survival in cancer patients (259). Interestingly, blockade of PD-1 decrease the suppressive effect of Treg and enhanced the cytotoxic function of effector cells (261).

1.2.5.4 *DC vaccines*

Given that DC are the most efficient APC, preclinical studies have been stressed to generate DC-vaccines in vitro. Monocyte-derived dendritic cells (mDC) generated in vitro, have been a great tool for generating DC-vaccines. Observations from independent studies, clarify that naturally occurring human mDC require IL-4 or GM-CSF to differentiate to mDC in vivo (262-264). Therefore such cytokines has been used in vitro to differentiate blood monocytes (265).

In the last two decades maturation of blood monocytes to fully matured mDC in vitro has been driven for production of therapeutic vaccines for the benefit of cancer patients (266-270). Briefly, blood monocytes are differentiated to immature DC by IL-4 and GM-CSF following a maturation stage where different cytokines or TLR-agonists are utilized and loaded with tumor-specific antigens (271, 272). Production of therapeutic DC vaccines has been focusing on inducing effective anti-tumor immune responses in T cells. Although DC can elicit anti-tumor T cell responses (270, 273-275), the clinical benefit for patients with cancer has been marginal (276, 277).

1.3 NK CELLS IN THE CLINIC

1.3.1 Targeting KIR

There are numerous of studies to enhance NK cell anti-tumor activity against cancer. Initial efforts in the 1980s were done to infuse LAK (lymphokine activated killer cells)/NK cells in cancer patients with or without IL-2 administration, where few clinical responses were seen in these patients, thought to be paralyzed by the tumor immune-suppression (278-280). Today no complete tumor rejection has been shown following NK cell infusion in patients with solid tumors (281-283). However, more successful adoptive immunotherapy with NK cells has been presented in patients with hematological malignancies (284, 285). One of the central mechanisms that have been used in these successful therapies is avoiding the interaction of inhibitory receptors KIRs with cognate HLA and therefore selecting “mismatched” clones for adoptive transfer of NK cells (285-288). Therefore it is of high importance to assess the tumor phenotype if possible before NK cell infusion, to predict the susceptibility of tumors to NK cell lysing to select more beneficial NK cell clones or to select suitable patients for specific therapy.

Such studies showing the clinical benefit of using mismatched NK cell clones emerged the idea of developing monoclonal antibodies to block KIRs. Completed phase I studies of anti-inhibitory KIR mAb IPH2101 in acute myeloid leukemia and in multiple myeloma, indicates a safe profile in the patients and can block KIR for prolonged periods of time with limited side-effects (289, 290). Evaluating NK cell activity in these patients identified roughly response parameters such as increased expression levels of CD69 and partially CD25 and elevated serum levels of TNF- α and the NK cell and monocyte chemoattractant MIP-1 β (**Figure 11**).

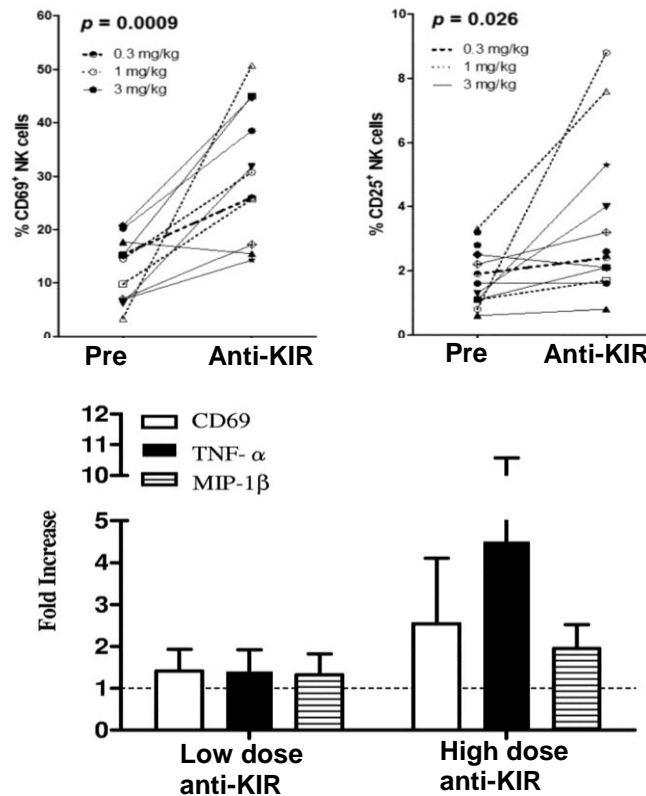


Figure 11: Clinical trial with anti-KIR. NK cell expression levels of CD69 and CD25 were analyzed by flow cytometry and serum levels of TNF- α and MIP-1 β were measured by ELISA. The figures are modified from original figures in *Blood* 2012;120:4324-4333 and *Blood*. 2012 Nov 22;120(22):4317-23.

1.3.2 Sensitizing tumor cells to NK cell-mediated killing

Other therapies to render tumor cells susceptible to NK cells targeting have been suggested. Rituximab or Mabthera are specific chimeric antibody for CD20 have been developed to target B-cell lymphomas (291). Combinatorial studies with mAB and IL-2 showed that such treatment can mediate NK cell ADCC targeting of the tumor cells (292, 293). Therapies on augmenting death receptor-mediated killing of tumor cells by NK cells have recently been performed. Several in vitro and in vivo studies have shown that tumor cell killing is mediated by NK- or T-

cell TRAIL interaction. Pharmaceutical manipulations like proteasome inhibitors, anthracycline antibiotics, and histone deacetylase inhibitors, increase tumor susceptibility to NK cells by through enhanced TRAIL-R expression (294-298).

1.3.3 Improve NK cell anti-tumor activity

Resting NK cells have no or very low expression of death ligands (TRAIL and FasL). Therefore, efforts to up-regulate expression of death ligands on NK cells in order to augment the cytotoxic function of NK cells should be considered. Administration of cytokines , like IL-2, not only results in increased expression of death ligands on NK cells, but also support NK cell proliferation and cytotoxicity in vivo such as IL-2 (299-301). Substances such as Zoledronic acid and Lenalidomide have been shown in vitro to increase TRAIL expression on NK cells and that correlate with increased cytotoxicity (302, 303).

Pre-conditioning has been shown to improve NK cell activity both in mouse-models and in patients. Such treatment can be radiation therapy and/or chemotherapy. Such treatment increases NK cell recognition of the tumor cells by upregulating for instance stress ligands, make space for NK cells to proliferate and induce NK cell responses to the standard treatment (304, 305).

Combining several strategies to enhance the activity and reduce the suppression in the tumor microenvironment is essential for efficient NK cell-based immunotherapy.

2 AIM OF THE THESIS

The general aim of my thesis is to study NK cell activity in the tumor microenvironment.

The study is divided into two parts; 1) how to augment NK cell activity against tumor cells, and 2) examine the suppression of NK cells in the tumor microenvironment.

Part 1

- I. Pharmaceutical manipulations of established tumor cell lines to increase their susceptibility to NK- and T cell-mediated killing in vitro and in vivo. Enforcement of TRAIL-receptor expression on tumor cells**
- II. Uncover novel strategies to increase TRAIL expression on NK cells to improve NK cell cytotoxicity against tumors**

Part 2

- III. Examine the interaction between NK cells and dendritic cells. Investigate the inhibitory effects of DC on NK cells. Develop new DC-vaccines to activate NK cells**
- IV. Study the NK cell interaction with myeloid-derived suppressor cells. Examine mechanisms by which MDSCs inhibit NK cell anti-tumor activity**

3 RESULTS AND DISCUSSION

Immunotherapy against cancer has in the last 20 years become a field of great interest. Increased understanding of how NK cells recognize and kills malignant cells has sparked investigators to explore the therapeutical role of NK cells in patients with cancer. Nonetheless, tumor cells can escape innate and adaptive immunity and thereby limit the outcome of NK cell-based therapies. Aiming to increase the insights into the mechanisms applied by tumors to avoid immune responses has provided a good understanding of the complex system of the tumor microenvironment. Below is a description of four individual papers included in this thesis and discussion of the findings.

Paper I: A novel inhibitor of proteasome deubiquitinating activity renders tumor cells sensitive to TRAIL-mediated apoptosis by natural killer cells and T cells

Tumor cells with a low or absent expression of MHC class I can be targeted by NK cells while tumor cells with a high expression of MHC class I can be targeted by T cells. Therefore, to maximize the effect of the immune system, it is of a great interest to develop a therapy of combined NK and T cell infusion. We hypothesized that chemotherapy agents that can sensitize tumors to NK and T cell mediated anti-tumor immune responses with combined immunotherapy may result in better clinical responses in patients with cancer (**Figure 12**).

We initially screened several human tumor cell lines following treatment with variety of already identified chemotherapy agents and pre-clinical compounds that have potential to sensitize tumor cells. The treatments were divided to short-term and long-term exposures (3-72 hours) at physiological doses. Following treatments, tumor cells were analyzed for expression of diverse membrane-bound recognition proteins including; TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), MICA/B, Nectin-2, PVR, Fas, and MHC class I. We found two potential drugs that up-regulate mainly TRAIL-R2 and slightly increased TRAIL-R, and some of the stress ligands. I focused on a substance called b-AP15. b-AP15 is a proteasome inhibitor inhibiting the regulatory subunit of the proteasome compared to bortezomib that regulate the core unit 20S (306, 307). Such inhibition of 20S

can mediate disturbances in the antigen presenting process which in turn inhibit the activation of T cells (308). Cells treated with b-AP15 accumulate polyubiquitinated proteins, with similar kinetics to cells treated with bortezomib. b-AP15-induced proteasome inhibition was concomitant with the up-regulation of apoptotic markers and the accumulation of cell-cycle regulatory proteins, leading to cell cycle arrest and apoptosis independent of the tumor suppressor p53 or overexpression of the BCL2 oncogene, which are implicated in bortezomib resistance (307, 309). Further we investigated if tumor cells were more sensitive to killing by NK and T cells following treatment with b-AP15 in vitro and in tumor bearing mice. NK cells were isolated and expanded from peripheral blood mononuclear cells (PBMC) of healthy donors and melanoma patients. Tumor specific T cells were isolated and expanded from tumor biopsies from HLA-A2 positive melanoma patients.

Human tumor cells treated with b-AP15 were significantly more sensitive to killing by NK cells and tumor (Mart1) specific T cells. When neutralizing different killing pathways, TRAIL-mediated killing was confirmed to be the major mechanism used by both NK cells and T cells to target b-AP15 treated tumors.

In vivo, pre-treatment with b-AP15 resulted in augmented T and NK cell of tumors in both immune deficient and immune competent tumor-bearing mice. Thus, targeting tumors with both the innate and adaptive arm of the immune system might lead to beneficial anti-tumor effects in patients with cancer.

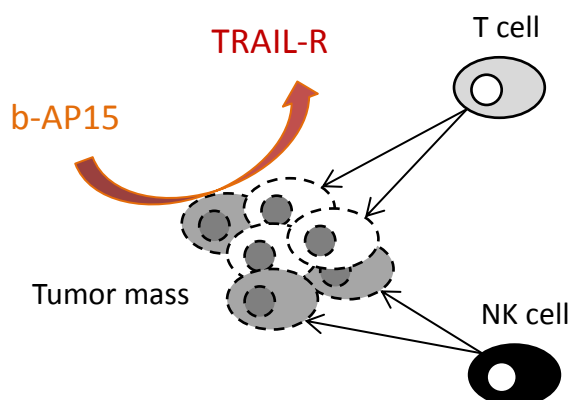


Figure 12: Tumor cells with heterogeneous expression of MHC I are easier to target by combination of NK cells and T cells. We show that treatment with b-AP15 increases tumor susceptibility to NK and T cell killing by upregulating TRAIL-R2 expression.

Paper II: Activated monocytes augment TRAIL-mediated cytotoxicity by human NK cells through release of IFN- γ

Based on the findings of Paper I, where expression of TRAIL on NK cells is an important factor for optimal tumor killing, we sought to investigate whether the expression of TRAIL could further be increased on NK cells.

In this paper we investigated if exposure to Zoledronic acid (ZA) would increase the expression of TRAIL on human NK cells and lead to increased TRAIL-mediated killing of tumor cells. Treatment of purified human NK cells with ZA had no effect on the TRAIL expression. However, treatment of PBMC with ZA resulted in increased TRAIL expression on NK cells. Thus, accessory cells within the PBMC were needed to induce TRAIL expression on NK cells when treated with ZA. In a series of experiments we concluded that treatment of monocytes with ZA resulted in increased production of IFN- γ . Upon neutralization of IFN- γ in co-cultures on monocytes and NK cells in presence of ZA, the expression of TRAIL was reduced to background levels (**Figure 13**).

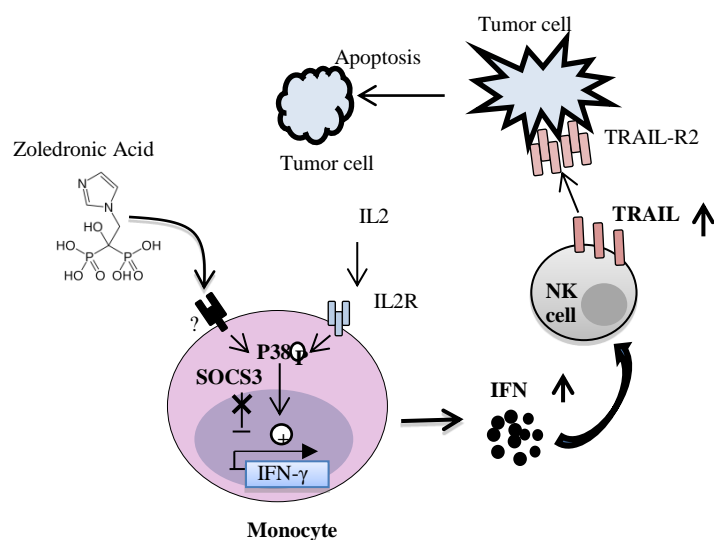


Figure 13: ZA induce a cascade of signals in monocytes which result in IFN- γ production which in turn upregulate TRAIL on NK cells. These NK cells are highly cytotoxic against tumor cells.

Next we investigated the anti-tumor activity of ZA-primed NK cells in tumor-bearing mice. Human NK cells were cultured with autologous monocytes in presence or in absence of ZA prior infusion. Mice treated with ZA-primed NK cells had significant slower tumor progression and prolonged survival compared with unprimed NK cells.

In conclusion, we show that ZA and IL-2-treated monocytes augment TRAIL-mediated cytotoxicity by human NK cells. We further identify IFN γ as the main factor responsible for the increased TRAIL expression on human NK cells. In a recent randomized phase III study, adjuvant therapy with ZA showed no clinical benefit in patients with breast cancer (310). On the other hand, another study showed that treatment with ZA in combination with IL-2 resulted in sustained serum levels of TRAIL, which correlated with improved clinical outcome in patients with hormone-refractory prostate cancer (311). Our findings suggest a new strategy to augment NK-cell anti-tumor activity by ex vivo exposure to ZA and IL-2, and infusion of such NK cells could potentially result in improved clinical outcome in patients with cancer.

Paper III: Regulation of natural killer cell responses by dendritic cells via lymphotoxin-alpha, interleukin-12, and tumor growth factor-beta

Driven by the findings in Paper II, we decided to investigate whether also monocyte-derived DC would activate NK cells in the presence of ZA. Intriguingly, we found that the NK cell activity was impaired in presence of DC compared with when cultured alone. While several studies have shown increased NK cell activity in pre-clinical murine models of DC vaccination, much less is known about the role of DC vaccines in shaping natural killer (NK) cell responses in patients with cancer. To study NK cell responses following interaction with DC in vivo, we collected blood from chronic lymphatic leukemia (CLL) and melanoma patients before and after DC-vaccination. Compared to pre-vaccination, the expression of the activation receptors NKp30/46 and CD16 was significantly reduced on NK cells in post-vaccination samples from patients with CLL. We next investigated the mechanisms of DC-mediated suppression of NK cell responses. We were able to confirm the previous findings showing that DC enhance proliferation, cytotoxicity, and IFN- γ production in resting NK cells. However, in presence of IL-2, DC

suppressed these NK cell responses. IL-2 increases phosphorylation of STAT-3 and high phosphorylation status has been reported to be associated with negative regulation of DC function (312). Therefore, we next inhibited STAT-3 in DC prior to co-culture with NK cells. In these conditions, DC did no longer suppress NK cell activity. We further found that suppression of NK cell activity by DC was independent on contact. We next analyzed production of cytokines by DC and STAT-3 inhibited DC and found a pronounced increase of IL-12, lymphotoxin-alpha (LTA), and down-regulation of TGF- β production by STAT-3-inhibited DC compared with untreated DC. These three cytokines act in concert to influence the activity of NK cells. TGF- β reduced the production of IL-12 and LTA in co-cultures of NK cells and DC and IL-12 or LTA was essential for maintained NK cell activity. Taken together, we conclude that the cross-talk between DC and NK cells is regulated through production of LTA, IL-12, and TGF- β (**Figure 14**). It might be of high importance to manufacture DC with low STAT3 phosphorylation to avoid suppression of NK cell responses.

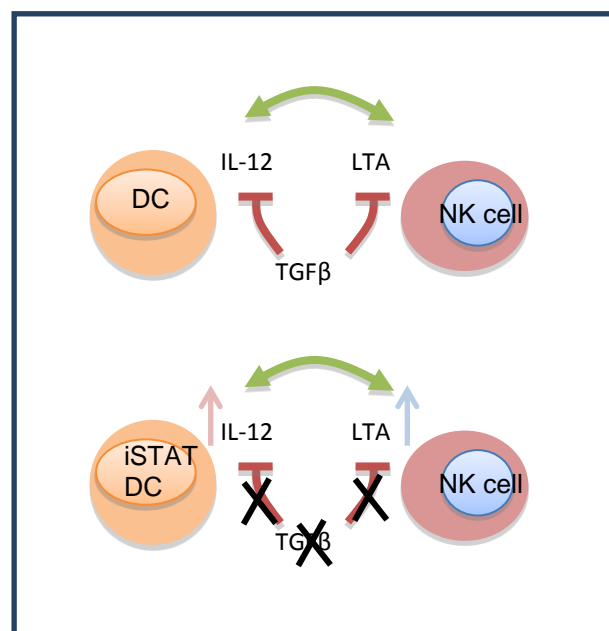


Figure 14: NK-DC crosstalk before and after STAT3 inhibition.

Paper IV: Myeloid-derived suppressor cells inhibit NK cell activity through prostaglandin-E2 regulated TGF- β production

In this study we investigated the role of MDSCs on NK cells. We previously developed a co-culture system where COX-2 expression by tumor cells was essential to convert monocytes into MDSC-like cells (223). Here we treated monocytes with recombinant PGE2 to induce MDSC-like cells. PGE2 bound to the EP2 and EP4 receptors resulting in decreased expression of HLA-DR, and increased levels of phosphorylated p38MAPK and ERK, and increased production of TGF- β . These cells indeed suppressed the activity of NK cells. Similar to patient-derived MDSCs, the PGE2-treated monocytes suppressed NK cell responses by production of TGF- β (**Figure 15**).

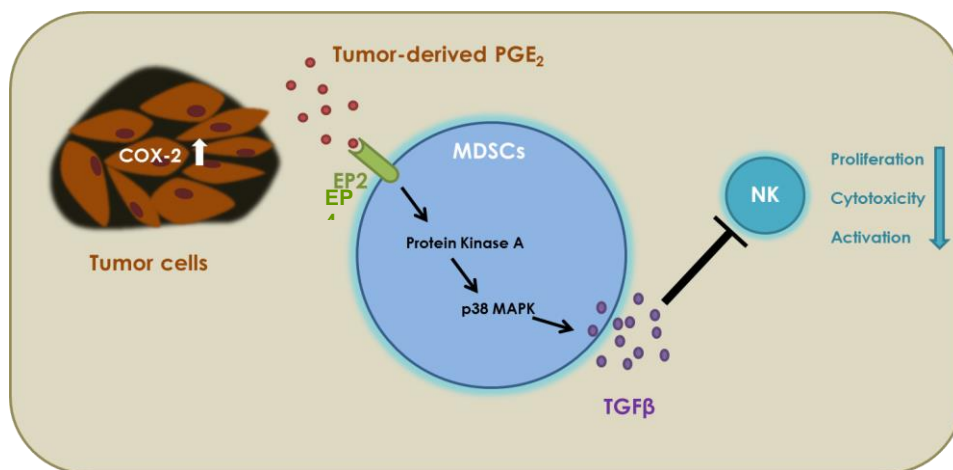


Figure 15: Mechanism of MDSC induction and suppression of NK cells.

To assess the functional consequences of COX-2 expressing tumors in vivo, we used a murine model, where control or COX-2-silenced 4T1 mammary carcinoma cells were s.c inoculated. Significantly higher percentage of CD11b⁺Gr1⁺ MDSCs and lower percentages of NK cell population were observed in mice inoculated with COX-2 silenced 4T1 cells compared with wt 4T1 cells. Furthermore, mice bearing COX-4 silenced 4T1

tumor had better rejected NK cell-sensitive YAC-1 cells compared with mice bearing wt 4T1 cells. Taken together, our data demonstrated a novel role of PGE₂ in inducing suppressive functions of MDSCs on NK cells, primarily by activating the production of TGF- β in myeloid cells through EP2/4 receptor and p38MAPK/ERK pathway. Given the prevalence of COX-2 over-expression and the central immune-regulatory role of TGF- β , these mechanisms might be relevant in several types of human cancers. Consequently, combining COX-2 inhibitors or EP antagonists with adoptive NK cell therapy may consolidate and enhance the clinical benefits for cancer patients.

4 OVERALL CONCLUSIONS

Although NK cells are “natural” in their targeting of tumors, by reading this book I hope I have convinced you that determining their responses in the tumor microenvironment is no bed of roses. As a tumor immunologist I believe in the immune surveillance theory, though the failure of the immune system to eliminate tumors may be in part due to sustained immunological selection pressure on tumor cells resulting in the development of tumor escape variants that are effectively invisible to the immune system. The tumor microenvironment is a complex network consisting of many different cells and factors that all contribute to diminishing the immune effectors to eliminate cancer. The miserable have no other medicine but only hope (William Shakespeare). About immunotherapy we have not only hope, we have clinical evidences that claiming our movement towards effective immunotherapy in cancer patients.

In this thesis new strategies to efficient NK cell-based therapy have been suggested and new findings about understanding the mechanisms of suppressing NK cells in tumors have been revealed (**Figure 16**). To be suppressed or activated?

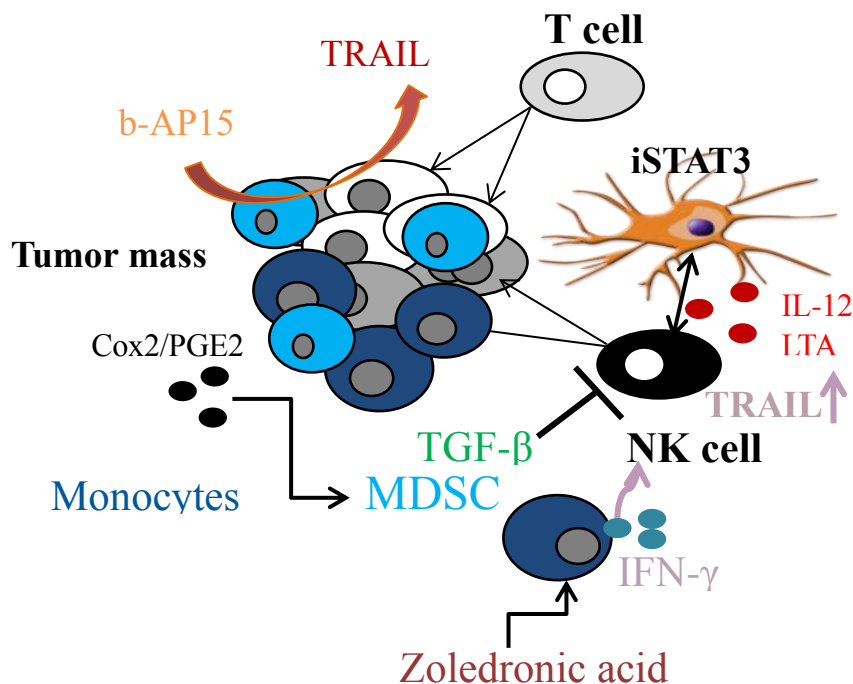


Figure 16: Overall achievement of the thesis.

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